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Persistent Chlamydiae: from Cell Culture to a Paradigm for Chlamydial Pathogenesis

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INTRODUCTION

The obligate intracellular bacterium *Chlamydia trachomatis* is an important human pathogen responsible for blinding ocular disease (trachoma) in developing countries and for sexually transmitted diseases throughout the world. The disease process associated with chlamydial infections is thought to be principally immunologically mediated. Repeated episodes of infection clearly play a role in stimulating the host immune response and enhancing damaging pathologic changes. It is unclear, however, if reinfection alone or the presence of an altered form of chlamydiae, capable of persisting in an inapparent state, also contributes to the resulting pathologic changes. Persistent chlamydial infections have been established in a number of short-term cell culture systems. These model systems reveal deviations in the pattern of chlamydial development, with viable organisms temporarily arrested in a nonproductive state of the growth cycle. Prolonged delays in the natural progression of development suggest an innate ability of *Chlamydia* species to persist intracellularly and may ultimately represent a facet of their role in the disease process. Identification of factors associated with altered chlamydial growth may also improve our understanding of chlamydial disease, including aspects of diagnosis, prolonged immunopathologic manifestations, sequelae, and effective treatment. This review evaluates a variety of cell culture model systems for chlamydial persistence and the biochemical and structural attributes corresponding to altered chlamydial development. The several modes of achieving persistent chlamydial infection in cell culture and the corresponding features of these infections will be evaluated and correlated to hallmarks of natural chlamydial disease.

DEVELOPMENTAL CYCLE OF CHLAMYDIAE

Chlamydiae have successfully developed an obligate intracellular lifestyle, overcoming obstacles of entry into a host cell, subsistence and propagation inside the host cell, and survival during transit in a hostile extracellular environment. To overcome these obstacles, chlamydiae have evolved a developmental cycle encompassing alternating functional and morphological forms (122). The elementary body (EB) is the infectious form of the organism, responsible for attaching to the target host cell and promoting its entry. The small, dense EB has a rigid cell wall conferred by extensive disulfide cross-linking of the major outer membrane protein (MOMP) and two cysteine-rich proteins: the 60-kDa envelope protein and the 12-kDa outer membrane lipoprotein (28, 29, 42). Interestingly, chlamydiae are among the few prokaryotes that do not have a cell wall composed of peptidoglycan (6, 31), although cross-linking components analogous to pentapeptide structures may be present since chlamydial growth and division are altered in the presence of β-lactam antibiotics (59, 67). The structural integrity of EBs confers resistance to environmental factors, thus permitting survival after lysis of the host cell and during subsequent transit from cell to cell as well as from host to host. In contrast, the reticulate body (RB) is the larger, metabolically active form of the organism, synthesizing DNA, RNA, and proteins. RBs are less-rigid, highly labile forms that do not survive outside the host cell (114). EBs and RBs, which are distinct forms of chlamydiae, appear to represent evolutionary

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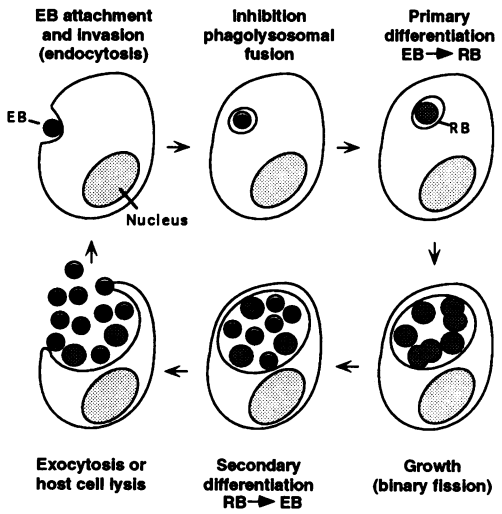

FIG. 1. Schematic diagram of the *Chlamydia* developmental cycle.

adaptations to extracellular and intracellular environments, respectively.

The chlamydial growth cycle is initiated when an infectious EB attaches to a susceptible host cell, promoting entry into a host cell-derived phagocytic vesicle (Fig. 1). The metabolically inert EB undergoes morphological changes, reorganizing to the larger RB. The resulting RBs are metabolically active, dividing by binary fission within the expanding endosome, which becomes visible as a microcolony that is referred to as the chlamydial inclusion. After a period of growth and division, RBs reorganize, condensing to form infectious EBs. The developmental cycle is complete when host cell lysis or exocytosis of chlamydiae occurs, allowing the EBs to initiate new infectious cycles. The length of the complete developmental cycle, as studied in cell culture models, is 48 to 72 h and varies as a function of the infecting strain, host cell, and environmental conditions.

Like many obligate intracellular pathogens, chlamydiae are capable of eluding normal defense mechanisms of the host cell. The endocytosed chlamydiae are sequestered within a host-derived phagosome during the intracellular phase of the developmental cycle. Inhibition of fusion with host lysosomal vesicles allows for intracellular growth and survival of these organisms. The prevention of phagolysosomal fusion appears to be directed by chlamydial surface antigens and to be dependent on the presence of viable chlamydial EBs (12, 126).

Control and Regulation of Intracellular Development

Although the life cycle of chlamydiae is well characterized by microscopy, the signals that trigger interconversion of the morphologically distinct forms are unknown. The substantial alterations in structural and biochemical characteristics that occur during the growth of chlamydiae suggest the existence of environmentally controlled regulatory events. Following entry into the host cell, primary differentiation of EBs to RBs encompasses alterations in the outer membrane structure, with reductive cleavage of the extensive disulfide cross-linking between MOMP and other outer membrane proteins (OMPs) (43). Covalent cross-linking of these outer membrane proteins contributes to the structural integrity of the rigid EB (9, 42). Reduction of disulfide bonds results in increased membrane fluidity and porin activity, allowing for enhanced permeability, nutrient transport, and metabolic activity (9). The absence of extensive cross-linking also accounts for the osmotically and mechanically fragile nature of RBs (34). Although disulfide-mediated interactions of chlamydial OMPs coincide with the conversion of EBs to RBs, this event alone does not trigger differentiation. When chlamydial EBs are exposed to reducing conditions, a number of changes occur that are associated with differentiation to RBs (34). Treatment of EBs with dithiothreitol results in a reduction in disulfide linkages with subsequent increased permeability and metabolic activity, decreased osmolarity and infectivity, and differential staining characteristics of RBs (34). However, the chlamydiae retain discrete, compact, electron-dense nucleoid structures characteristic of EBs, suggesting that membrane structural reorganization alone is not sufficient to induce differentiation. In RBs the DNA is loosely packed, with diffuse fimbrils extending throughout the cytosol. Changes in the structural organization of the nucleoid appear to be imperative to the developmental progression of chlamydial growth. The decondensation of the chlamydial chromosome may be essential for transcriptional and translational activation. As the infectious process progresses, differentiation of RBs to infectious EBs is accompanied by reincorporation of MOMP and other OMPs into outer membrane complexes (43). These events occur concomitant with the dramatic recondensation of the chlamydial chromosome.

The actual mechanisms that control and regulate intracellular development are unknown. Identification of temporally regulated proteins implicates an orderly system of developmental regulation in chlamydiae. Several stage-specific proteins are synthesized as early as 15 min postinfection (89). Synthesis of a number of proteins is associated transiently with primary differentiation, while other proteins continue to be produced at low levels throughout the late log phase of development (61, 89, 124). Early-stage proteins identified include a homolog of bacterial glutamyl-tRNA synthetase (124), the chlamydial S1 ribosomal protein, a GroEL-like protein, and a DnaK-like protein (61). The GroEL and DnaK proteins are both stress response proteins. Although their exact functions are not known, these proteins have been implicated in folding, unfolding, and translocation of proteins, as well as in assembly and disassembly of oligomeric complexes (27). Early synthesis of these proteins precedes that of MOMP, a protein constitutively expressed throughout the intracellular growth of chlamydiae (112). Synthesis of these stress proteins may play a role in the early survival and differentiation of EBs.

Several late-stage-specific genes are expressed during the transition of RBs to EBs. These include those that encode envelope proteins (28, 34, 42, 43, 79) and DNA-binding proteins (7, 33, 85). Synthesis of the cysteine-rich structural proteins commences when RBs initiate reorganization to EBs (42, 79). Synthesis of a developmentally regulated protein with homology to eukaryotic histone H1 proteins was identified late in the developmental cycle and coincides with active chlamydial DNA synthesis and compaction of the chlamydial genome (7, 33, 85). A pattern of chlamydial protein synthesis consistent with structural and functional characteristics of chlamydial development has emerged. Regulation of intracellular development may be in response to environmental changes associated with the chlamydial growth cycle.

Metabolic Capacity

In adapting to an intracellular lifestyle, chlamydiae have lost their own energy-producing systems while retaining considerable biosynthetic capacity. Elements of glycolysis, respiration,

and pentose biosynthesis have been identified in chlamydiae; however, these organisms lack the enzymes required for net ATP production (74). Intracellular development of chlamydiae depends on ATP obtained from the host cell and possibly on a number of other host-acquired high-energy metabolites. The growing RB acquires host ATP by the action of a chlamydial ATP-ADP translocase (41). Chlamydiae then hydrolyze ATP to ADP to generate a proton motive force that drives the transport of other host-supplied compounds. Although they possess all the cell machinery for prokaryotic DNA, RNA, and protein synthesis, chlamydiae are dependent on their hosts for many precursors such as nucleotides and amino acids (40). In addition, these organisms are in continuous competition with host cells for vitamins, nutrients, and cofactors.

Altered Chlamydial Development

Deviations from the typical developmental stages of chlamydiae suggest an inherent flexibility in the intracellular growth of this organism. Following entry into the host cell, an EB reorganizes to a single RB. This event is thought to be reciprocated upon reorganization, with each RB giving rise to a single infectious EB. Ultrastructural evaluation reveals that the intermediate form between these elements of development generally contains a single site of nucleoid condensation. However, reorganization has been associated with multiple sites of DNA condensation capable of giving rise to multiple EBs (25, 122). In addition, reorganization and compartmentalization of RB membranes further suggests the generation of multiple EBs from a single RB (67, 122). The exact nature of host-supplied factors and the environmental conditions for typical chlamydial development are unknown. However, deviations from the typical developmental cycle correspond significantly to a variety of alterations in growth conditions, including the presence of antibiotics (3, 21, 49, 59, 67, 78, 119) or host-elaborated factors (10, 66, 107) and deviations in the levels of essential nutrients (1, 25). These conditions generally delay RB maturation, inhibit differentiation to infectious EBs, and are associated with gross morphological alterations of RBs typified by markedly enlarged, atypical chlamydial forms. Delays in chlamydial development in response to exogenous factors suggest an innate ability of these organisms to persist intracellularly in a unique developmental form (Fig. 2).

Persistent Chlamydial Development

"Persistence" describes a long-term association between chlamydiae and their host cell in which these organisms remain in a viable but culture-negative state. The term "persistent infections" implies the absence of overt chlamydial growth, suggestive of the existence of chlamydiae in an altered state distinct from their typical intracellular morphologic forms. Although persistence describes a long-lasting association that may not necessarily manifest as clinically recognizable disease, it is distinct from inapparent infections, which may or may not involve evident chlamydial growth. The terms "inapparent infection," "subclinical infection," and "asymptomatic infection" have been used almost interchangeably to indicate chlamydial infection in the absence of clinical manifestations. The term "inapparent" also may be used in defining the state of the chlamydial organism, connoting clinical disease in the absence of a diagnostically recognizable etiological agent. This definition of inapparent infection with reference to the bacterial organism is synonymous with persistent infection, with the exception that persistence emphasizes a more prolonged association.

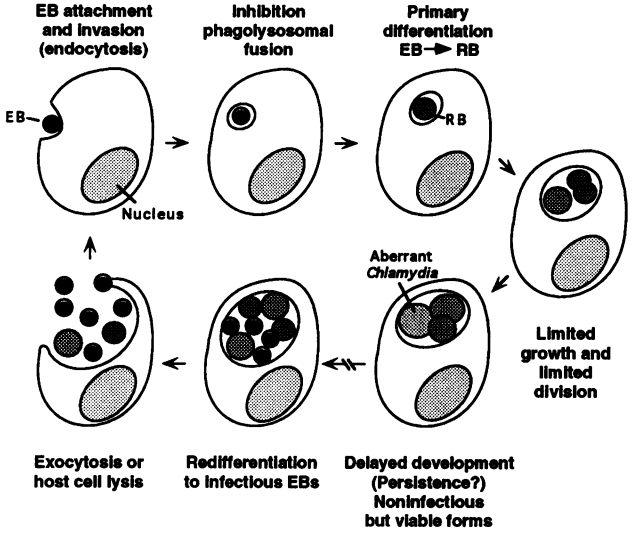

FIG. 2. Schematic diagram of altered intracellular *Chlamydia* development.

Parallels may be drawn between persistence as described for chlamydial infections and viral latency. Despite the vast and obvious differences in the infectious agents involved, both of these terms describe an interaction with host cells in which the progression of the agent through the usual developmental cycle is interrupted. Arrested growth of chlamydiae may correlate to a reduction in metabolic activity which restricts growth and division and delays differentiation to cultivatable EBs, therefore establishing a culture-negative state. Limited metabolic capacity may also influence the biochemical and antigenic attributes of the persisting organisms, potentially rendering them undetectable by normal diagnostic tests. In latent infections, infectious viruses are not recovered until some event occurs that reactivates viral development. A similar situation is proposed to occur in persistent chlamydial infections, such that conditions evolve that allow for viable dormant organisms to resume active growth and reorganization to infectious forms. Therefore, persistence may represent a deviation from the typical development of chlamydiae, resulting in delayed intracellular growth under the influence of exogenous factors that may not be as "typical" as cell culture growth conditions.

PERSISTENT INFECTION IN CELL CULTURE

The concept of persistent chlamydial infections is not new. Persistence, i.e., the perpetuation of chlamydiae within the host cell without overt growth or replication, has long been recognized as a major factor in the pathogenesis of chlamydial disease (69). The biological state of chlamydiae and the factors that lead to persistence in vivo are unknown; however, several studies have established cell culture systems to define parameters associated with the growth and induction of persistent chlamydial infections. Early work analyzed conditions for the maintenance of cell cultures chronically infected with chlamydiae for periods exceeding 1 year (30, 80). A stable relationship was established in which cell growth, damage, and chlamydial production were balanced. It was not surprising to early investigators that chlamydiae tended toward a persistent state, since chlamydiae were perceived as viruses and viral latency was commonly observed and intensely studied during this time.

It was not until it became obvious that chlamydiae actually were bacteria (75) that work directed toward understanding the specific details of how these organisms entered a persistent relationship with their host cell was scrutinized. It was clear that since chlamydiae exhibited true prokaryotic attributes, latency no longer served as an accurate description of chlamydial persistence and the persistence of these organisms must be quite different from true viral latency.

The presence of persistent chlamydiae intracellularly in an altered morphological form was first suggested by Moulder et al. (77). Infection of mouse fibroblasts (L cells) with *C. psittaci* at high multiplicities of infection results in alternations between periods of host cell destruction by chlamydial multiplication and periods of proliferation. The coexistence of L cells and *C. psittaci* can be maintained indefinitely in the absence of typical chlamydial inclusions as observed by microscopic examination. This unrecognizable form of chlamydiae was defined as a cryptic body. Host cells that survive an initial productive infection are resistant to superinfection by the same or heterologous strains and carry cryptic chlamydial forms as indicated by the occasional emergence of new productive infectious cycles. Host cell resistance to reinfection is attributed to altered surface protein profiles. Persistently infected cultures are susceptible to rifampin and chlortetracycline, indicating that active transcription of chlamydial DNA and translation of the resulting mRNA continue in these cryptic forms. However, the inability of penicillin and minocycline to cure L cells of persistent infection suggests that cryptic chlamydiae might be less susceptible to certain antibiotic therapy. Early studies that defined cryptic chlamydiae did not attempt to identify and characterize the cryptic or persistent form. However, each of these studies implied that cryptic forms were atypical in some way as a result of interruptions in the normal growth cycle of chlamydiae.

Nutrient Deficiency-Induced Persistence

A number of initial studies established persistent infections by maintaining infected host cells in nutrient-deficient medium (4, 5, 71). Chlamydiae are able to invade the deficient host cells and upon entry remain in a noninfectious but viable state. Addition of complete medium stimulates growth and recovery of infectious chlamydiae (4, 5). The importance of various amino acids and vitamins in chlamydial development was analyzed to define specific metabolites necessary for the conversion of a persistent infection to a state of active chlamydial growth. Persistence of *C. psittaci* in L cells occurs when infected cells are incubated in medium supernatant from 24-h cultures of uninfected cells (spent medium) (39). The presence of chlamydiae could not be demonstrated by either Giemsa stain or culture for infectivity. Persistence of the organism in a nonreplicating, noninfectious form is reversible by the addition of fresh medium, which promotes activation of chlamydial multiplication. Addition of L-isoleucine, an amino acid essential for both chlamydial and host cell growth, is sufficient to trigger normal chlamydial development. Persistent or productive chlamydial growth is dependent on the competition between the host cell and the intracellular bacterium for this single metabolite. Inhibition of host cell protein synthesis by the addition of cycloheximide prevents competition from the host cell, leaving the limited levels of isoleucine available for chlamydial protein synthesis and subsequent productive chlamydial development.

Depletion of cysteine interrupts chlamydial RB-to-EB differentiation in 10 different serovars of *C. trachomatis* and 3 strains of *C. psittaci* (1). This effect is reversible, with resumed differentiation to infectious forms, upon the addition of cysteine. The effect appears to be specific for cysteine, since the omission of other amino acids has little or no effect on chlamydial development. These observations suggest that an alteration in growth and differentiation arises via the requirement for this amino acid for the biosynthesis of three cysteine-rich proteins needed for RB-to-EB differentiation.

More recently, intracellular development of *C. trachomatis* serovars E and L2 and three strains of *C. psittaci* in medium lacking all 13 amino acids was analyzed (25). Reduction in the amino acid concentrations results in reduced infectious yield, with the development of enlarged abnormal chlamydial forms. This effect is reversible, with a substantial recovery of infectivity upon the reintroduction of amino acids. In contrast to earlier studies, omission of any 1 of the 13 amino acids, with the exception of valine, from the growth medium induced aberrant chlamydial development.

The above studies suggest that factors that compromise the biosynthetic capacity of the host cell potentially have an amplified effect on intracellular chlamydiae. Under conditions in which the host's soluble pools for metabolic needs become limited, chlamydiae may fail to successfully compete for macromolecular precursors and hence may enter a state of arrested growth. Alterations in the extracellular environment that stimulate host cell proliferation may raise intracellular pools, reactivating persistent chlamydiae to a state of productive developmental growth.

Antimicrobial Agents and Persistence

A number of studies have analyzed antimicrobial agents as mediators of persistent chlamydial development. Treatment with penicillin has no effect on initial differentiation of the infecting EB to the RB but prevents the process of binary fission, inducing the development of enlarged, morphologically abnormal chlamydial forms with a block in the production of progeny EBs (67). These aberrant forms have been found in both *C. psittaci*- and *C. trachomatis*-infected cultures treated with penicillin (3, 21, 24, 49, 59, 67). Ultrastructural analysis reveals that the RB forms become progressively larger with continued culture in the presence of the antibiotic, with masses of membranes forming outside and inside the swollen RBs (67). Removal of penicillin from the extracellular culture medium results in extensive budding and internal subdivision of the aberrant chlamydial forms, producing typical RBs (67) with maturation to infectious EBs (30).

Unequal cellular division, with the formation of small daughter RBs, termed "miniature" RBs, budding both within and from enlarged chlamydial forms, is another feature observed with penicillin treatment (115). In vivo features similar to those observed with penicillin were identified in ultrastructural analyses of *C. trachomatis*-infected mouse oviduct (88). A similar observation was made in cell culture studies of *C. psittaci*-infected cells treated with DEAE-dextran or cycloheximide (110). Pyknotic forms develop within aberrant RBs when the cells are treated with these two very different reagents.

Because chlamydiae are deficient in peptidoglycan (6, 31), the mechanism of chlamydial growth inhibition by penicillin is unknown (76). In *C. trachomatis* LGV-infected cultures, penicillin has no effect on the synthesis of MOMP; however, the synthesis of the cysteine-rich 60-kDa envelope protein, an integral component of structural integrity, is completely inhibited (21). Ampicillin also blocks the conversion of RBs to EBs (98). The effect of ampicillin on chlamydia-specific protein synthesis reveals that arrest of chlamydial development is accompanied by a marked inhibition in the expression of both

60-kDa and 12-kDa cysteine-rich proteins, while the production of MOMP is only minimally affected (98).

D-Cycloserine, also an inhibitor of peptidoglycan synthesis, has a growth-inhibiting effect on chlamydiae. In *C. psittaci* MoPn-infected cells treated with this antibiotic 16 h after infection, the chlamydiae appear as abnormally large, swollen forms similar to those observed after penicillin treatment (78). Growth inhibition of *C. psittaci* by D-cycloserine can be competitively reversed by its structural analog, D-alanine.

Chloramphenicol and chlortetracycline have also been shown to interrupt the intracellular development of chlamydiae (59, 119). The effect of these inhibitors of prokaryotic protein synthesis is dependent on the stage during infection at which the chlamydia-infected cells are exposed to these antibiotics. Addition of these inhibitors early in infection prevents primary differentiation of EBs to RBs, whereas exposure later in infection interrupts RB division and secondary differentiation. A nearly identical situation occurs when the effect of erythromycin on chlamydial development is analyzed (23). This macrolide antibiotic not only inhibits RB-to-EB differentiation but also induces notably smaller inclusions containing RBs of approximately twice the diameter of typical RBs. Erythromycin binds to the 50S subunit of the ribosome and may reduce RB ribosomal activity and subsequent protein synthesis. Decreased protein synthesis may result in a deficiency in membrane constituents or regulatory proteins required for differentiation.

Antibiotics that inhibit nucleic acid synthesis also have been shown to inhibit chlamydial development. 5-Fluorouracil affects late stages of the *C. trachomatis* LGV growth cycle (3). Cells treated with 5-fluorouracil for 24 h appear similar to untreated cultures. However, by 48 h, smaller inclusions containing only a few noninfectious larger particles are present. When *C. psittaci*-infected fibroblasts are cultured in the presence of nalidixic acid, an inhibitor of DNA gyrase, binary fission of RBs and subsequent reorganization to EBs are inhibited (119). Hydroxyurea, another inhibitor of DNA synthesis, also interrupts the developmental cycle of *C. trachomatis* by reversibly inhibiting the transformation of RBs to infectious EBs (94). As observed with penicillin and ampicillin, this interruption in RB-to-EB differentiation is accompanied by the inhibition of the synthesis of cysteine-rich proteins (98). Given the disparity in the molecular targets of these drugs, the reduced synthesis of cysteine-rich proteins may be secondary to alterations in regulatory signals which trigger RB-to-EB differentiation.

Sulfonamides also have been demonstrated to alter chlamydial morphology and development in cell culture (35, 36, 93). Most *C. trachomatis* strains are susceptible to the antagonists of bacterial dihydrofolate reductase, whereas most *C. psittaci* strains are resistant (50, 60, 100). Treatment of *C. trachomatis*-infected McCoy cells with trimethoprim or sulfomethazole at levels slightly below the MIC inhibit RB-to-EB differentiation characterized by the presence of gross changes in RB formation (35, 36). By 48 h postinfection, inclusions contain irregular RBs, with ruffled membranes, mini-RB-like forms, and numerous ghost particles (36). Culturing *C. trachomatis*-infected cells with increasing concentrations of both trimethoprim and sulfomethazole results in inclusions that are progressively smaller, pyknotic, and less abundant (35). When cells containing these abnormal inclusions are transferred to drug-free medium, the morphology of subsequent inclusions is normal.

Persistent infection of McCoy cells with *C. psittaci* can be achieved by the addition of aminopterin within 20 h of infection (90). Aminopterin inhibits dihydrofolate reductase, preventing conversion of dihydrofolic acid to tetrahydrofolic acid. Cultures lacking visible inclusions and infectivity can be maintained for 4 weeks, with chlamydial replication resuming upon addition of folinic acid.

A vast number of antibiotics inhibit chlamydial differentiation. The effects of different antibiotics seem to be dependent on the level present and the stage during the developmental cycle in which infected cells are exposed to these substances. When comparing the alteration of chlamydial growth by these antibiotics in tissue culture, the primary molecular target for each is vastly different. This suggests that there is not a single mechanism by which differentiation is inhibited and persistence is generated.

Immunologically Induced Persistence

Abnormal or persistent chlamydiae have been recognized under a variety of artificial cell culture systems, but only recently have reasonable physiological mediators of persistence been identified and the biochemical and antigenic features of persistent chlamydiae characterized. Most of the work in this area has focused on immune system-regulated cytokines that do not directly affect chlamydiae but, rather, cause alterations in the host cell, interfering with normal intracellular chlamydial growth. The most extensively studied of these cytokines is the T-cell product gamma interferon (IFN-γ).

Chlamydiae were among the first nonviral pathogens reported to induce IFNs and were shown to exhibit growth inhibition in their presence (68). Inhibition of intracellular chlamydial growth by IFN in cell culture systems was reported by several groups (26, 38, 57, 95). At the same time that these studies were being done, independent work focused on immune system-induced lymphokines and their inhibiting effects on intracellular chlamydial growth in macrophages (14, 15). The active component in supernatant fluids from stimulated T cells was identified as IFN-γ (16, 97), which activated not only mononuclear phagocytes (97) but also fibroblasts (16) and epithelial cells (17) to restrict intracellular chlamydial replication. IFN-γ is a macrophage-activating factor which induces microbicidal products of oxygen metabolism. However, oxygen-dependent mechanisms do not contribute to the restriction of chlamydial growth (96, 106). Rather, IFN-γ was found to inhibit *C. psittaci* growth in a human epithelial cell line by inducing indoleamine 2,3-dioxygenase, a nonconstitutive enzyme that catalyzes the degradation of tryptophan (17). This mechanism has previously been implicated in activating host cells to restrict the obligate intracellular protozoan *Toxoplasma gondii* (87). Depletion of exogenous tryptophan correlates with growth inhibition of both *C. psittaci* and *C. trachomatis* in a wide spectrum of human host cells including primary conjunctival epithelial cells (92), the actual host cell that is infected during chlamydial ocular disease.

While high levels of IFN-γ completely restrict chlamydial growth, low levels induce the development of morphologically aberrant intracellular forms (106). These studies suggested that IFN-γ treatment of host cells might lead to maintenance of chronic chlamydial infection. More recent studies have characterized the ultrastructural features associated with IFN-γ-mediated persistent *C. trachomatis* infection (10). IFN-γ treatment of *C. trachomatis* serovar A-infected cells results in the development of enlarged, atypical, noninfectious forms distinct from both EBs and RBs (10) and morphologically similar to those induced by penicillin treatment (59, 67) (Fig. 3). These persistent organisms not only exhibit a highly unusual intracellular morphology but also display differential expression of key chlamydial antigens (10, 11) with continued

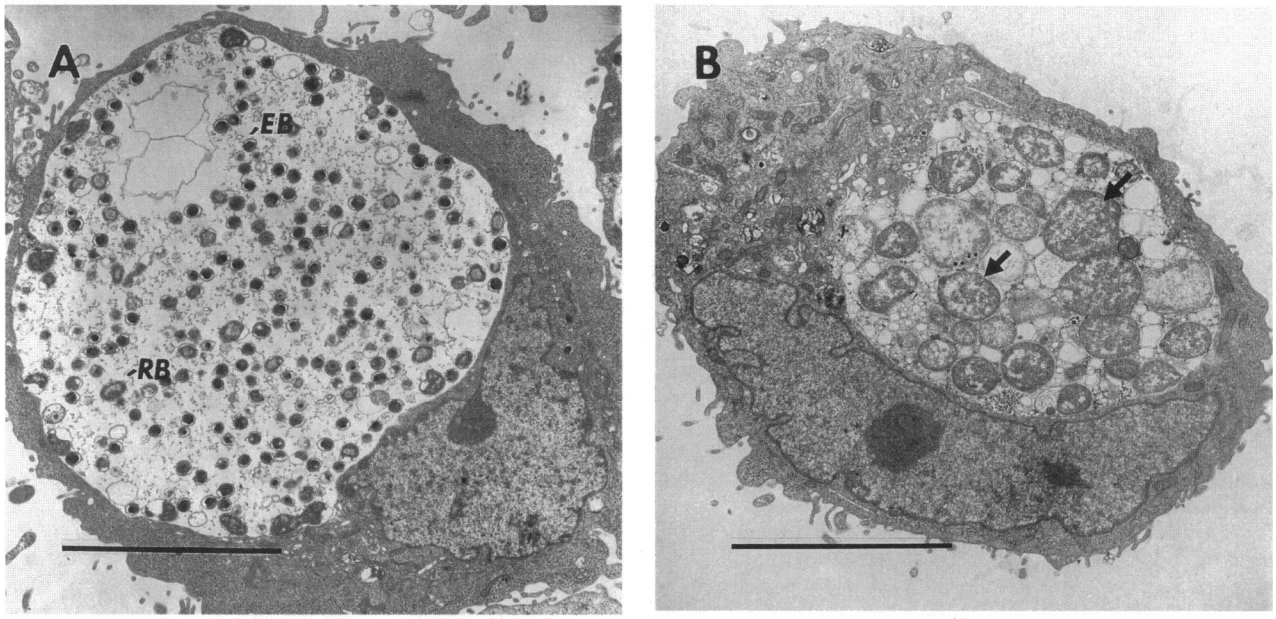

FIG. 3. Electron micrographs of *C. trachomatis*-infected cells. (A) A typical inclusion, 48 h postinfection, containing EBs and RBs. (B) IFN-γ treatment of infected cells, resulting in smaller inclusions containing enlarged, atypical RB forms (arrows). Bars, 5 μm.

synthesis of hsp60, an immunopathological antigen (73), and reduced synthesis of MOMP, a protective antigen (113, 129). In addition, these forms exhibit a reduction in the levels of other structural constituents of chlamydiae, including the 60-kDa envelope protein and lipopolysaccharide (10).

The term "persistence" implies a prolonged association between chlamydiae and their host cells. IFN-γ-mediated persistent infection can be maintained for several weeks in cell culture, with reactivation of viable infectious organisms following the removal of IFN-γ at any time during the experimental time course (11). It was not known if recovery of infectivity was a result of renewed growth and differentiation of typical RBs contained within the population of persistent chlamydiae or whether the persistent organisms themselves could reorganize in some fashion to produce infectious EBs. If the former hypothesis is true, it would imply that the persistent state is a dead-end pathway and not a true alternative mode of chlamydial development. In contrast, if the latter hypothesis is true, persistent organisms would retain the ability to undergo reactivation to infectious forms. Studies were done to distinguish between these two hypotheses (11). The development of infectious chlamydiae from aberrant organisms is characterized by internal reorganization within the enlarged forms, extensive budding processes, and miniature-RB formation (Fig. 4), with subsequent emergence of morphologically typical EBs (11). Multiple nucleoid-like masses within the aberrant forms following IFN-γ removal are similar to intrachlamydial structures observed under nutrient-deficient conditions (25). These observations suggest that endopolygeny, in which several progeny are generated from a single aberrant chlamydial form, may occur.

Studies that demonstrate a role for IFN-γ in activating host cells to restrict subsequent chlamydial growth require relatively large amounts of IFN-γ, with host cells treated for at least 24 h prior to infection to achieve optimal growth inhibition (17, 105, 106). Persistence of *C. trachomatis* occurs when very low levels of IFN-γ are added to culture systems following infection of the host cells (10, 11). The differences in experimental design required to induce persistence as opposed to growth inhibition are probably more representative of conditions that exist during an actual chlamydial disease, that is, the presence of infected cells prior to the release of IFN-γ by activated T cells and a relatively modest level of IFN-γ at the site of infection.

Morphological features associated with IFN-γ-mediated persistence have similarities to those of abnormal growth induced by penicillin. It is striking that such completely different mediators lead to abnormal organisms that share many of the same characteristics, further legitimizing these aberrant forms. The fact that an immune system-regulated cytokine can cause these alterations provides a reasonable physiological mechanism for their induction in vivo. The antigenic changes that accompany reactivation of penicillin-treated organisms have never been characterized, but it would not be surprising if similar changes to those reported for IFN-γ-induced persistence were observed.

Not only does intracellular growth of chlamydiae in the presence of penicillin induce persistence, but also infected cells release chlamydial hsp60, which upon purification mediates an ocular delayed-hypersensitivity response in immune guinea pigs (72). If secretion of hsp60 also occurs in response to IFN-γ, this would have further implications in the stimulation of immunopathologic changes following infection and the development of immune system-mediated chlamydial persistence. Evidence that persistent chlamydial growth is associated with an increase in the expression of chlamydial hsp60 (11) suggests that persistent growth of chlamydiae ultimately may be a stress-related response. As anticipated, heat shock of *Chlamydia*-infected cells results in enlarged aberrant forms typical of penicillin and IFN-γ treatment (53).

Other immune system-mediated factors may induce similar alterations in chlamydial development and contribute to persistent growth. HEp-2 cells infected with *C. trachomatis* serovar L2 display a decrease in both the production of chlamydial DNA and the infectious yield when cultured with monocytes or monocyte-derived macrophages (66). The inhibitory effect of

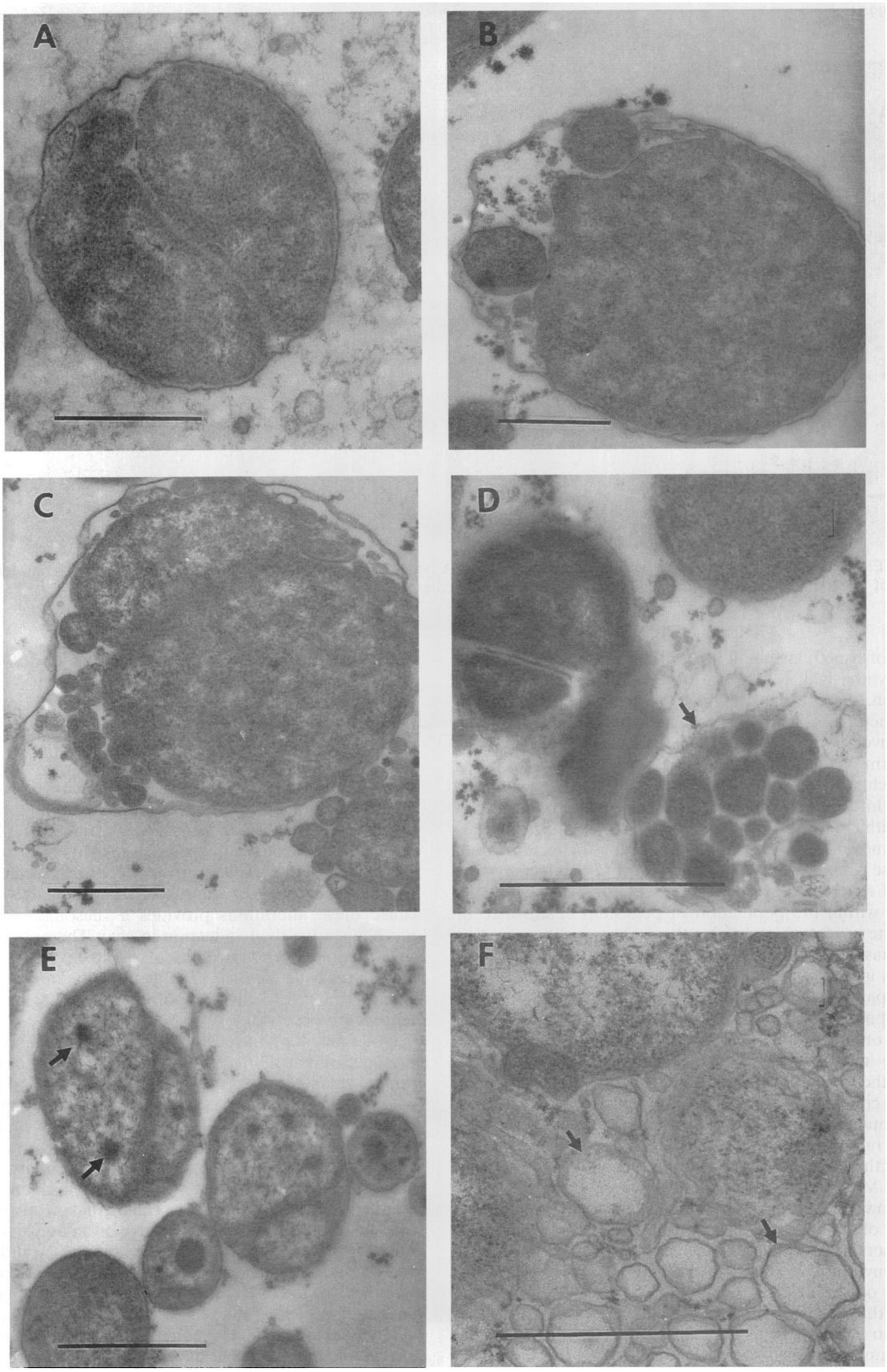

FIG. 4. Features associated with alterations in the typical *Chlamydia* developmental cycle. (A) Large RBs, displaying internal fragmentation. (B and C) Extensive budding from enlarged RBs (B) and "miniature" RB formation (C). (D) Reorganization and development of numerous chlamydial forms within a single membrane (arrow). (E) Multiple sites of DNA condensation (arrows) within a single RB, indicating the development of multiple EBs from a single RB. (F) Development of numerous membrane structures or ghosts (arrows) within the chlamydial inclusion. Bars, 1 μm.

monocyte-derived macrophages is more pronounced, with ultrastructural analysis of infected cells revealing smaller inclusions containing primarily enlarged reticulate-like bodies and very few typical EBs. This effect is not dependent on cell-to-cell contact between the two populations as demonstrated by continued inhibition in the presence of an artificial membrane (99). Addition of antibodies to tumor necrosis factor alpha (TNF-α), an immune factor produced by macrophages, reduces the inhibitory effect of these cells on chlamydial DNA production. In addition, TNF-α alone inhibits the growth of *C. trachomatis* serovar L2 in HEp-2 cell cultures (107). Ultrastructural evaluation reveals that infected cells treated with high levels of TNF-α during early stages of infection contain relatively smaller inclusions with irregular, enlarged RBs. Inhibitory growth is partially reversed by simultaneous addition of TNF-α and monoclonal antibodies to IFN-β, suggesting that IFN-β plays a mediatory role in the antichlamydial effect of TNF. In addition, the inhibitory effect of TNF-α on the growth of *C. trachomatis* is effectively suppressed by elevated levels of tryptophan.

Other Potential Mediators of Persistence

Changes which indirectly affected chlamydiae via alteration of host cell signal transduction pathways may suffice to induce abnormal chlamydial developmental forms. Chlamydial development may be regulated by cyclic nucleotides, with cyclic GMP (cGMP) stimulating chlamydial growth and cAMP being inhibitory (123). Treatment of *C. trachomatis* L2-infected cells with cAMP reversibly interferes with the correct progression of chlamydiae through the developmental cycle (55, 56). Inclusions are small and immature and remain noninfectious in the presence of cAMP. In addition, a study of *C. trachomatis* persistence in BHK cells suggested that levels of exogenous cAMP may be important in reactivating persistent organisms to overt infection (65).

Ca^{2+}, another intracellular mediator regulating cellular activities, was also shown to be important in the growth and development of chlamydiae. Treatment of *C. trachomatis*-infected cells with the calcium antagonist verapamil interfered with chlamydial development, inhibiting RB-to-EB differentiation (104).

The chlamydial Mip-like protein (macrophage infectivity potentiator), a homolog of eukaryotic and prokaryotic FK506-binding proteins, exhibits peptidyl-prolyl isomerase (PPIase) enzymatic activity (62, 63). Inhibitors of PPIase activity, FK506 and rapamycin, reduce chlamydial infectivity when the organism is pretreated with these drugs or exposed early in infection (63). Prolonged exposure for intervals from 0 to 24 h postinfection resulted in small, morphologically abnormal inclusions containing large, swollen structures similar to those seen when infected cells are treated with IFN-γ or penicillin. FK506 and rapamycin interact with FK506-binding proteins, blocking the interaction of these proteins with their natural ligands and subsequently inhibiting signaling events. It is not clear if the aberrant chlamydial development arises from inhibition of PPIase activity of the chlamydial Mip-like protein or from complete suppression of host PPIase, which indirectly affects the chlamydiae.

Interruptions in the typical growth cycle of chlamydiae have been recognized for decades and are associated with abnormal developmental forms. The vast number of factors associated with altered growth suggests that the "normal" chlamydial growth cycle, as defined in cell culture, may not be the only process of chlamydial development, especially in the complex environment of a natural infection.

Summary of Alterations Associated with In Vitro Persistence

Several studies have characterized inhibition of chlamydial growth by microscopy and found it to be associated with an alteration in the typical chlamydial developmental cycle such that unusual forms with properties distinct from both EBs and RBs emerge. The distinct features associated with altered chlamydial development are shown in Fig. 4. These unique alterations have been identified by using antibiotics, nutrient-deficient conditions, and immune system-regulated factors (3, 10, 25, 49, 59, 67, 78, 107, 115). Further characterization of this alteration to aberrant chlamydial growth reveals that several factors inhibit the generation of infectious progeny, although the abnormal organisms remain viable (10, 30). In addition, penicillin, ampicillin, hydroxyurea, cAMP, IFN-γ, and cysteine depletion have all been implicated in impairment of the synthesis of one or more of the key structural components of chlamydiae (1, 10, 21, 55, 98).

Normally, chlamydiae undergo an orderly alternation between two developmentally regulated forms, the infectious EB and the metabolically active RB. Late in the developmental cycle, redifferentiation of the RBs to the infectious EBs is accompanied by the synthesis and incorporation of MOMP and other OMPs into outer membrane complexes (42). Thus, division and differentiation appear to be dependent on the availability and appropriate cross-linking of these OMPs. Interruptions in the synthesis of outer membrane constituents may result in continued chlamydial growth without proper division and differentiation, accounting for inclusions containing fewer organisms that are morphologically enlarged. Alternately, a reduction in outer membrane integrity may allow for increased osmotic influx, accounting for the swollen chlamydial structures. In contrast, reduced levels of OMPs may be secondary to the suppression of signaling pathways that prevent binary fission of RBs and redifferentiation to EBs. The resulting decrease in the surface-to-volume ratio of enlarged atypical structures, compared with normal chlamydial RB and EB forms, would account for the decrease in structural outer membrane constituents.

PERSISTENCE OF CHLAMYDIAE IN VIVO

Chlamydial persistence has been established in a variety of cell culture systems; however, conclusive documentation of persistent chlamydiae in natural infections remains to be demonstrated. Indirect evidence indicates that such conditions occur and may contribute to the immunopathogenesis associated with both ocular and genital chlamydial diseases.

Evidence for Persistence in Ocular Infections

Although a clear correlation between trachoma and infection with *C. trachomatis* has been established, chlamydiae cannot be identified by tissue culture or immunocytological methods in over 20% of cases, even in the presence of severe abnormalities (116, 117). A variable range of clinical manifestations has been reported for the course of this disease. A subset of individuals with active chlamydial infection displaying moderate to severe trachoma had no detectable viable organisms (102). Other studies have identified individuals with severe scarring disease who were positive immunocytologically but were culture negative (64). The converse situation has also been described in cases involving ocular infections in which chlamydial inclusions were found in the absence of clinical signs of disease (37).

The diverse clinical manifestations of chlamydial ocular infection correlate to different stages of the disease process. In

areas where trachoma is endemic, early stages of the disease are characterized by mild clinical symptoms, generally associated with younger individuals experiencing active infection as detected by the presence of cultivatable chlamydiae (32). Older individuals who have been exposed to repeated infections display complications of ocular scarring and fibrosis, corneal trauma, and blindness, even though *C. trachomatis* is rarely isolated from the diseased tissue (32). Progressive scarring disease with clinically inapparent, nonproductive (culture-negative) infection indicates that these individuals may harbor a cryptic form of chlamydiae in the infected tissue. The presence of such quiescent chlamydial forms is further suggested by studies of individuals who left areas where trachoma was endemic and had not experienced active disease since childhood but developed acute trachoma several decades later (101). Therefore, viable chlamydiae may be present in a latent, nonreplicating form, inconspicuously contributing to the progression of disease toward blinding trachoma.

Strong evidence for the continued presence of *C. trachomatis* in a culture-negative state comes from studies of trachoma patients and a primate model for ocular disease in which the presence of unamplified chlamydial rRNA in conjunctival swabs was demonstrated long after antigen detection was negative (22, 46, 48). Because RNA molecules are very labile, identification of chlamydial RNA indicates that chlamydiae may persist in diseased tissue in a culture-negative but viable form. Chlamydial rRNA also has been detected in the synovium of patients with Reiter's disease, in the absence of cultivatable chlamydial organisms, suggesting that intact, culture-negative organisms persist in the joints (91).

These studies indicate that viable chlamydiae may be present at the site of infection and induce an inflammatory response beyond the time when standard techniques can detect the organism. However, confirmation of the contribution of chlamydial persistence to the disease process awaits identification of latent but viable chlamydiae in natural infection.

Evidence for Persistence in Genital Infections

The identification of *C. trachomatis* as an etiological agent of infertility in women is well recognized. Infection of the cervix by *C. trachomatis* may be chronic, persisting unrecognized for months to years unless a symptomatic infection of a male partner occurs. Approximately 70% of women with *C. trachomatis* cervicitis have only mild symptoms or remain asymptomatic (20). Unrecognized infection can progress, ascending to the salpinges and fallopian tubes and resulting in pelvic inflammatory disease. A large proportion of *C. trachomatis* infections of the fallopian tubes are also asymptomatic or subclinical, influencing the potential for the development of the adverse sequelae of infertility and ectopic pregnancy. Silent infections are the most common cause of tubal infertility, with only one-third of infertile women having a history of pelvic inflammatory disease (103, 118). Several studies indicate a strong association between serological evidence of a previous *C. trachomatis* infection and obstructive infertility (13, 44, 45, 51, 70, 84, 108); however, recovery of viable chlamydiae from tubal biopsy specimens is rare (13, 54, 70, 118). Few studies have demonstrated isolation of *C. trachomatis* from damaged tissue of the upper genital tract (44, 45); in one study, multiple passages in culture were required for isolation of infectious organisms (108). Whether chlamydiae persist subclinically in the upper genital tract of women, with resulting infertility, is unclear and remains a matter of debate.

Although chlamydiae are difficult to culture, especially when the disease process has become chronic, the presence of chlamydial antigen and nucleic acids is indicative of persisting organisms. A number of studies have identified *C. trachomatis* antigen in endometrial and tubal specimens of culture-negative infertile women (18, 58, 118). Chlamydia-specific DNA has been identified in endocervical cells and fallopian tube tissue of women with tubal occlusion (18, 109). Only a fraction of samples positive for chlamydia-specific DNA were also positive by immunocytological techniques for the chlamydial MOMP, an antigen detected in diagnostic tests for chlamydiae (18, 109).

There are several possible explanations for why the presence of chlamydial antigens, resulting in immunologic stimulation and disease, may continue in the absence of cultivatable organisms. Residual chlamydial antigens and DNA may remain from a previous, completely eradicated infection. However, because of the prolonged course of these diseases, it seems improbable that long-term chronic inflammation and disease could be attributed to nonreplicating chlamydial antigen residing in the diseased tissue. The inability to verify the presence of *C. trachomatis* by routine culture may indicate that such procedures are not sensitive enough to detect low but immunologically significant levels of chlamydiae. In addition, the presence of neutralizing antibody or host-elaborated metabolic inhibitors may interfere with detection by culture. Alternatively, affected individuals may harbor a persistent form of chlamydiae that possesses the characteristics of noncultivatable RBs but retains viability and the capacity to stimulate immunopathologic changes. However, evidence of chlamydial antigen and nucleic acids in the absence of cultivatable organisms only suggests the possibility of persisting chlamydiae (18, 22, 118). These studies have not examined patients over time; therefore, there is no direct evidence for a long-term culture-negative state in patients with *C. trachomatis* genital infections.

Mediators of Reactivation in Natural Infection

Experimentally induced persistence indicates that a number of factors can arrest intracellular chlamydial development from which infectious organisms cannot be isolated. Removal of these factors leads to a resumption of active growth of chlamydiae and development of infectious progeny. Reactivation of apparently resolved chlamydial infection in vivo occurs under conditions of immunosuppression, implicating the immune response in maintenance of persistent infection (120, 127). A number of physiologic environmental stimuli may reactivate such quiescent infections to generate a prolific growth state with the production of infectious, immunologically significant forms. The epidemiological relationship between *Neisseria gonorrhoeae* and *C. trachomatis* suggests that concurrent infection may play a role in reactivation of inapparent chlamydial infections (8, 81). Chlamydiae are isolated in significantly greater numbers from women with gonorrheal infection than from those without (83). In addition, women exposed to partners with gonorrhea are more likely to yield chlamydiae if they become infected with *N. gonorrhoeae* than if they do not (83). In addition to gonococcal infection, trauma to the genital tract is thought to stimulate mucosal turnover, potentially triggering the conversion of persistent chlamydiae to an active growth phase (19). With the constant fluctuation in environmental conditions, a number of undefined factors (immune factors, nutrients, and hormones in genital infections) may stimulate a productive infection.

CORRELATIONS BETWEEN CELL CULTURE MODEL SYSTEMS AND EVENTS OCCURRING IN NATURAL INFECTIONS

In cell culture, 48 to 72 h following infection, lysis of the host cell can result in the release of hundreds of chlamydiae, each capable of eliciting a new round of infection. It is presumed that the chlamydial developmental cycle progresses in a similar manner during natural infection. Such a sequence of events would result in the rapid progression of the infectious process. However, chlamydial infections tend to exhibit a prolonged course, being insidious and asymptomatic. Although there is no direct evidence for altered chlamydial development in vivo, it is possible that chlamydial growth in natural infections is more complex, with interruptions in chlamydial differentiation and production of infectious progeny similar to events defined in cell culture models for persistence.

Chlamydiae infect mucosal epithelial cells; however, it is improbable that this is the site of persistent infection, because of the natural turnover of these target cells. Natural turnover and lysis of sloughed cells would result in release of chlamydial antigens and stimulation of the immune response but also in loss of persistently infected cells. However, recent evidence indicates that *C. trachomatis* may reside in the subepithelial tissue (18), thus allowing chlamydiae to persist for extended periods. It is likely that persistence of chlamydiae in natural infections encompasses a number of events to allow for maintenance of a long-term association. This includes alternations between the development of metabolically inactive persisting forms and periods of low-level multiplication. In addition, a balance established with the host cells may allow for continued multiplication of these cells, which in turn may result in cell-to-cell transfer of persistent chlamydiae at host cell division without lysis and immune stimulation.

Persistence of different biotypes of *C. psittaci* was thought to reflect differences in the capacity to enter a persistent relationship with their hosts during a natural infection (86). This idea is significant in that it suggests distinctions between clinical syndromes that are both strain and host cell related. If *C. trachomatis* has similar distinctions, it is possible that ocular and genital strains will exhibit different behaviors. Analysis of the amino acid requirements for *C. trachomatis* growth reveals that all strains tested require leucine, phenylalanine, and valine for normal growth (2). Genital serovars also require histidine and glutamine, while ocular serovars require all of these plus tryptophan (2). This requirement for tryptophan only by ocular strains is significant to the studies of IFN-γ-mediated induction of the persistence of *C. trachomatis* serovar A (10). The effects of IFN-γ on restricting the replication of genital *C. trachomatis* serovars may be governed by different IFN-γ-induced activities, or the effects of indoleamine 2,3-dioxygenase may be more complex than merely starvation of tryptophan. Stimulation by hormones, cytokines, or other factors may alter the growth of genital serovars, providing distinctions between the behavior of chlamydiae in different anatomical locations and disease syndromes.

Diagnosis of active chlamydial infection often entails sampling genital or conjunctival specimens for cultivatable chlamydiae. However, chlamydiae are often difficult to culture from diseased tissue, even though there may be evidence of progressive scarring (18, 109, 118, 121). This situation correlates with in vitro studies which show that chlamydiae can persist in host cells in abnormal forms that are noninfectious but retain viability (10). Immunofluorescent-antibody tests are used for direct examination of clinical specimens for chlamydial inclusions, typically utilizing antibodies to chlamydial MOMP and lipopolysaccharide. A number of factors shown to induce a persistent state in vitro are associated with a reduction in the levels of chlamydial MOMP (10, 55, 98), and lipopolysaccharide (10), indicating that if such an infection occurred in vivo, standard diagnostic immunofluorescent-antibody assays might lack the appropriate specificity to identify persistent forms of the organism. These studies may be helpful in establishing a rational basis for the development of alternative methods for demonstrating the presence of chlamydiae in cases of disease when culture and direct antigen detection methods prove to be negative. Cell culture model systems for persistence indicate that detection of infection may require the identification of enlarged, aberrant chlamydial forms within diseased tissue or diagnostic immunolabeling with antibodies to chlamydial antigens that continue to be synthesized in the persistent state, such as hsp60 (11).

Current available treatment regimens for *C. trachomatis* infections entail a 7- to 10-day course of tetracycline, erythromycin, or doxycycline (111). These multidose therapies have considerable potential for patient noncompliance, especially by individuals who are asymptomatic or display minimal symptoms. Inadequate doses or duration of therapy may result in incomplete clearance of the organism, with temporary inhibition of chlamydial growth, allowing for subsequent relapse. This prospect is analogous to cell culture studies assessing antichlamydial activity of different antibiotics. Eradication, as opposed to transient inhibition or induction of persistent chlamydial growth by antimicrobial agents, is contingent upon the duration of treatment, the dose, and the stage of the growth cycle during which chlamydiae are exposed to these agents.

Current clinical studies indicate that the antibiotic azithromycin is a promising single-dose therapy for *C. trachomatis* infections, potentially eliminating the problem of noncompliance (111). However, the prolonged course of *C. trachomatis* diseases and their tendency to progress asymptomatically reemphasize the potential of incomplete eradication, despite presumably appropriate antimicrobial therapy. A 7-day regimen of ciprofloxacin for treatment of *Chlamydia*-positive nongonococcal urethritis rendered patients culture negative after 2 to 3 days; however, *C. trachomatis* was reisolated within 4 weeks of the initial treatment (47). The recurrent strain in all cases had an identical serotype to the original infecting strain. In addition, there was a strong correlation between recurrences and the number of chlamydial infectious units present prior to treatment, suggesting that recurrent infection was a result of persisting organisms and not of reinfection. In contrast, a 7-day regimen of doxycycline effectively eradicated chlamydial lower genital tract infections, with individuals remaining negative by both PCR and culture for 5 months following treatment (125). The latter studies indicate that persistence of *C. trachomatis* may not be a factor in uncomplicated urogenital infections following appropriate antimicrobial therapy.

It has been suggested that early treatment of chlamydial infections is successful whereas antibiotic therapy during the chronic phase generally has little effect (82). There are several reasons to suspect that persistent chlamydiae may not respond to antibiotics as well as normally growing chlamydiae would. First, persistent chlamydiae may contain reduced levels of MOMP (10). MOMP not only acts as an antigen likely to stimulate protective immunity but also functions as a porin (9). In the absence of MOMP, large hydrophilic molecules, including many antibiotics, may not be transported into the chlamydial cell. However, currently there is no evidence that MOMP functions in the transport of drugs. Second, since persistence

appears to be a manifestation of a stress-related response, and since stress responses are known to induce phenotypic resistance to antibiotics in other bacteria (128), it might be expected that persistent chlamydiae would be less sensitive to antibiotics than normally growing organisms are. Finally, when clinical isolates of chlamydiae resistant to tetracycline were cultured, organisms growing in the presence of high levels of tetracycline exhibited a morphology strikingly similar to that of persistent chlamydiae as defined by the IFN-γ model (50). However, it is unclear if tetracycline is failing to eradicate a persistent infection or if the antimicrobial itself induces altered chlamydial growth (50, 52). If persistent chlamydiae are found to exhibit decreased susceptibility to antibiotics and if persistence can be documented in actual cases of chlamydial disease, these findings could profoundly influence the clinical treatment of chlamydial infections.

CONCLUSION

A significant contribution to the knowledge of chlamydial persistence comes from cell culture systems, but these models have obvious limitations. Animal models and epidemiological studies also provide evidence for the occurrence of chlamydial persistence; however, documentation of such a state in natural infections remains controversial. The number of exogenous factors reported to alter the typical progression of chlamydial growth suggests an innate flexibility in the intracellular development of this organism. In the complex environment of a natural infection, numerous host-elaborated factors with inhibitory or modifying effects may be present, resulting in delayed chlamydial development and subsequent persistence of this pathogen. Studies with host cells in culture that more accurately reflect in vivo host cells (primary conjunctival or endometrial cells) will provide additional clues to the interaction between chlamydiae and their host and induction of chlamydial persistence in a natural infection. Further studies will be helpful in establishing effective antibiotic usage strategies, as well as a rational basis for the development of alternate methods for the detection of persistent chlamydiae, when culture and direct antigen detection methods prove to be negative. However, confirmation of the contribution of chlamydial persistence to the disease process awaits the identification of latent but viable organisms in natural infection. The identification of persisting chlamydial organisms will impact our knowledge of the pathogenesis of chlamydial disease and provide a more complete (and possibly more accurate) understanding of the chlamydia-host cell interaction in natural infections.

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